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## DOSE DEPENDENCE AND THERAPEUTIC WINDOW FOR THE NEUROPROTECTIVE EFFECTS OF 17 $\beta$ -ESTRADIOL WHEN ADMINISTERED AFTER CEREBRAL ISCHEMIA

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### Abstract

The present study was undertaken to determine if the neuroprotective effect of 17 $\beta$ -estradiol (E<sub>2</sub>) when administered after ischemia is dose-dependent and if the therapeutic window for estrogen can be prolonged. Ischemic injury was induced by permanent middle cerebral artery occlusion (p-MCAO). Administration of E<sub>2</sub> at 30 minutes after ischemia resulted in a reduction in lesion volume. A higher dose of E<sub>2</sub> extended the therapeutic window to 6 hour after cerebral ischemia in 33% of the rats. These findings suggest that postischemic treatment with estrogen affords protection against ischemic damage and that it acts within a clinically useful therapeutic window.

### Keywords

Estrogen; Cerebral-ischemia; Neuroprotection

### Introduction

We initially reported that estrogens cause a dose-dependent protection of SK-N-SH cells under conditions of serum- deprivation [3,26]. Since these initial observations of *in vitro* neuroprotection with estrogen treatment, there have been several hundred reports describing the neuroprotective effects of estrogens [4,11]. This neuroprotection is seen in a variety of neuronal cell types in response to over 14 different neurotoxic insults *in vitro*, including serum-deprivation [14,26],  $\beta$ -amyloid [2,10] and excitatory amino acid treatment [5,10].

We have now extensively assessed the neuroprotective effects of estrogens in an animal model of cerebral ischemia. Following our first reports of neuroprotection with estrogens in an animal model of ischemia [25], we and other have demonstrated that estrogens protects the brain from ischemic damage induced by transient cerebral ischemia [15,21], permanent cerebral ischemia [6,30], subarachnoid hemorrhage [30], and global ischemia [13]. The protective effects of estrogens are seen with 17 $\beta$ -estradiol (E<sub>2</sub>), as well as non-feminizing estrogens, such as 17 $\alpha$ -estradiol [25], ENT-estradiol [12] and 2-adamantyl-estrone [16], suggesting that at pharmacological doses, estrogen receptors are not required for neuroprotection in stroke. Dubal et al. [9] reported that ER $\alpha$ KO, but not ER $\beta$ KO, mice were resistant to the neuroprotective

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effects of E<sub>2</sub> administered chronically at low physiological concentrations and concluded that ER $\alpha$  is a necessary mediator of estrogen neuroprotection. Later, however, Sampie et al. [21] observed that ER $\beta$ KO mice have unusually small infarcts, likely due to the extremely high levels of circulating E<sub>2</sub>, and McCullough et al [18] demonstrated estrogen neuroprotection in ER $\alpha$ KO mice using pharmacological doses of estrogens.

While much is known about the ability of pretreatment with estrogens to protect brain tissue from ischemia, few studies have assessed the effects of estrogens when administered after the onset of ischemia. We reported that the protection afforded by E<sub>2</sub> in ovariectomized rats when high doses are administered by an intravenous injects can be observed between 30 min and 3 hours following the onset of cerebral ischemia [25,32]. McCullough et al. [17] administered Premarin<sup>®</sup>, a conjugated equine estrogen preparation, to male rats after transient MCA occlusion and observed protection at 10 min, but not 90 min after the onset of occlusion. In the preliminary dose-finding study [31], with E<sub>2</sub> doses of 0, 100, 500, 1000, 2000 and 5,000  $\mu$ g/kg body weight when administered at 6 hours after the onset of the occlusion, we observed that there was a dose-dependent reduction in lesion volume from 100 to 500  $\mu$ g/kg body weight, a persistence of the effect at 1,000  $\mu$ g/kg body weight and a relative loss of protection at the higher two doses. As such, in the present study, we selected the optimal dose for delayed neuroprotection, 1000  $\mu$ g/kg body weight, using a subcutaneous preparation for rapid, sustained delivery of estrogens.

## Materials and Methods

### Experimental animals and E<sub>2</sub> preparation

Female Charles Rivers Sprague-Dawley rats (12 weeks old, Wilmington, MA) were acclimatized to animal facilities three days prior to surgery. Bilateral ovariectomy was performed 2 weeks before permanent middle cerebral artery occlusion (MCAO). All animal procedures were approved by the University of Florida and the University of North Texas Health Science Center Animal Care and Use Committees. E<sub>2</sub> was first dissolved in absolute ethanol. Then, sesame oil with 2% benzyl alcohol was added to the solution to yield the final concentration of 100  $\mu$ g/ml, 500  $\mu$ g/ml, or 1 mg/ml. The ethanol was evaporated off.

### E<sub>2</sub> serum concentrations

A group of animals (n=7) were ovariectomized to eliminate endogenous ovarian steroids. After two weeks, E<sub>2</sub> was injected subcutaneously (s.c.) at the dose of 100  $\mu$ g/kg. Blood samples were taken via a permanent atrial cannula prior to administration of E<sub>2</sub> (time 0), and at 5, 15, 30 and 60 min, and 4, 8, 24 hours after E<sub>2</sub> administration. Blood samples were centrifuged for 20 minutes and plasma was separated and stored at -20 °C until assayed. Plasma concentrations of E<sub>2</sub> were determined with an ultra-sensitive estradiol radioimmunoassay kit (Diagnostic Systems Lab, Webster, TX.).

### Cerebral ischemia

Two weeks after ovariectomy, animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). During the procedures, rectal temperature was monitored and maintained between 36.5 °C and 37 °C with heating lamps and warming pads. With the aid of an operating microscope, the left common carotid artery and internal carotid artery were exposed through a midline cervical skin incision. A 4-0 monofilament suture with a rounded-tip was introduced into the internal carotid artery via the external carotid artery lumen and advanced until resistance was encountered. The distance between the common carotid artery bifurcation and the resistant point was 2.2 cm. A 6-0 silk ligature was placed around the external carotid artery to prevent bleeding and movement of suture position. The

common carotid artery and pterygopalatine artery temporary ligatures were then released, and the skin incision was closed.

### Measurement of Lesion Volume

Animals were decapitated 48 hours after MCAO, and the brain was harvested and placed in a metallic brain matrix for tissue slicing. Seven 2 mm coronal slices were made at 3, 5, 7, 9, 11, 13, and 15 mm posterior to the olfactory bulb. Each slice was incubated for 30 minutes at 37 °C in a 2% solution of 2,3,5-triphenyltetrazolium chloride (Sigma, St. Louis, MO) followed by fixation in 10% formalin. Stained slices were photographed by a digital camera (Sony, Tokyo, Japan). The ischemic lesion area in both side of each slice was traced and subsequently calculated for ischemic lesion volume (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). The total ischemic lesion volume was calculated as the sum of the volume of the ischemic lesion across the 7 slices.

### Protocol 1

This study was conducted to determine if the neuroprotective effects of E<sub>2</sub> were dose-dependent when administrated 30 minutes after MCAO. A total of 21 rats were divided into 3 groups. The animals were treated either with a single subcutaneous injection of E<sub>2</sub> at the dose of 100 µg/kg (OVX+ E<sub>2</sub> 100 µg/kg, n=5) or 500 µg/kg (OVX+E<sub>2</sub> 500 µg/kg, n=7). For controls, ovariectomized females (OVX, n=9) were subcutaneous injected with equivalent volumes of the vehicle. The animals were decapitated 2 days after MCAO and the ischemic lesion volumes were determined.

### Protocol 2

Our previous study demonstrated that E<sub>2</sub> exerts neuroprotective effects when intravenously administrated up to 3 hours after an ischemic insult at a concentration of 100 µg/kg (43). Herein, we tested whether higher doses of E<sub>2</sub> could prolong this therapeutic window. E<sub>2</sub> was administered subcutaneously with the dose of 1 mg/kg at 30 minutes (OVX+E<sub>2</sub> 30 minutes group, n=6) or 6 hours (OVX+E<sub>2</sub> 6 hours group, n=12) after MCAO. Control animals received equal volume of vehicle at 30 minutes (OVX 30 minutes group, n=7) or 6 hours (OVX 6 hours group, n=8) after MCAO. The ischemic lesion volume was measured as described in protocol 1.

### Statistic analysis

Statistical analysis was performed using Prism software (GraphPad Software, Inc, San Diego, CA.). All data are presented as means ± SEM. Ischemic lesion volumes were compared by one-way ANOVA followed by Tukey tests. A probability of <0.05 was considered significant.

## Results

### Effect of E<sub>2</sub> on mortality

The permanent MCAO caused inadvertent death of some animals due to excessively large lesion volume. Deaths prior to the planned 48 hr sampling of animals occurred in 5 OVX and 4 E<sub>2</sub>-treated rats. These rats fully recovered from surgery and anesthesia, then died within 48 hours after occlusion. Interestingly, in this subset of animals, OVX rats displayed more severe motor deficits and died earlier (<12 h after the occlusion), while, the E<sub>2</sub>-treated rats had milder motor deficit and survived up to 24–36 h after occlusion. Due to the poor staining of the ischemic lesion, all the premature died animals were excluded from the lesion volume analysis.

### Plasma E<sub>2</sub> concentration after subcutaneous administration

Plasma estradiol increased rapidly after subcutaneous injection of E<sub>2</sub> (100 µg/kg), reaching over 2000 pg/ml within 5 min after administration. The plasma concentration of E<sub>2</sub> peaked around 1 hour post-injection, and decreased thereafter (Fig. 1). At 24 hours after administration, plasma E<sub>2</sub> was still elevated with a concentration of  $167.9 \pm 71.8$  pg/ml.

### Dose-dependent neuroprotective effect of E<sub>2</sub> when administered at 30 minutes after cerebral ischemia

Permanent occlusion of the MCA resulted in a mean total lesion volume of  $352 \pm 28$  mm<sup>3</sup>, with the lesion localized primarily in the parietal cortex ( $223 \pm 21$  mm<sup>3</sup>) and basal ganglia ( $130 \pm 12.0$  mm<sup>3</sup>) (Fig. 2 & 4). Subcutaneous E<sub>2</sub> administration at 30 minutes after permanent MCA occlusion significantly decreased the total lesion volume to  $224 \pm 21$  mm<sup>3</sup> and  $130 \pm 31$  mm<sup>3</sup> with the dose of 100 µg/kg or 500 µg/kg, respectively. This reduction corresponds to  $36 \pm 6\%$  and  $63 \pm 9\%$  of the control, respectively (Fig. 2A). The estrogen-mediated protection was observed in both cortex and subcortex regions (Fig. 2B & 2C). In the ischemic cortex, subcutaneous E<sub>2</sub> injection at the dose of 100 µg/kg or 500 µg/kg E<sub>2</sub> resulted in a mean cortical lesion volume of  $137 \pm 21$  mm<sup>3</sup> (a  $39 \pm 9\%$  reduction) or  $66 \pm 26$  mm<sup>3</sup> (a  $71 \pm 12\%$  reduction), respectively. In the subcortex, E<sub>2</sub> treatment decreased lesion volumes to  $84 \pm 6$  mm<sup>3</sup> (a  $35 \pm 5\%$  reduction) at the dose of 100 µg/kg, and  $73 \pm 9$  mm<sup>3</sup> (a  $44 \pm 7\%$  reduction) at the dose of 500 µg/kg.

### Dose dependent therapeutic window of E<sub>2</sub> protection

Ischemic lesion volumes were significantly reduced from  $352 \pm 28$  mm<sup>3</sup> in controls to  $212 \pm 24$  mm<sup>3</sup> (a reduction of  $40 \pm 7\%$ ) in E<sub>2</sub> treatments when E<sub>2</sub> was administered with the dose of 1 mg/kg at 30 minutes after ischemic insult. When administered at 6 hours after occlusion, protective effect of estrogen was demonstrated in 33% of animals tested (4 of 12 rats) with the dose of 1 mg/kg. In this subgroup, the ischemic lesion volume was reduced from  $318 \pm 29$  mm<sup>3</sup> in control group to  $136.4 \pm 28.6$  mm<sup>3</sup> ( $57 \pm 9\%$  reduction) in E<sub>2</sub> treated group. However, protective effect of E<sub>2</sub> was not indicated in 67%, or 8 out of 12 rats, in the E<sub>2</sub> treated group when E<sub>2</sub> was administered with the dose of 1 mg/kg at 6 hours after the onset of MCAO.

## Discussion

The present study demonstrates 3 important features for the potential use of estrogens for the treatment of ischemic stroke. First, the protective effect of estrogen is dose-dependent when administered after the onset of cerebral ischemia. Second, the therapeutic window of E<sub>2</sub> appears to be at least 6 hours in a subgroup of animals. Finally, subcutaneous administration of an oil formulation of E<sub>2</sub> produces a rapid and sustained increase in circulating estrogens at the concentrations that are neuroprotective for at least 24 hours. Collectively, these data indicate that estrogen formulations and dosing regimens can profoundly protect the brain from ischemic damage.

The oil formulation tested in the present study produced a rapid increase in circulating E<sub>2</sub> concentrations, with high level seen by the first sampling time at 5 min. and levels were maintained above those needed to protect brain tissue [22,32] for at least 24 hours. In as much as no toxicity was seen with this formulation and mode of dosing E<sub>2</sub>, it appears to be a safe therapeutic. Indeed, even in animals that died as a result of large ischemic lesion produced, the E<sub>2</sub> treatment appears to improve symptoms and survival time.

Our observation of dose-dependent protection with E<sub>2</sub> administered after the onset of MCAO is important since the safety of E<sub>2</sub> have not been established for acute high dose administration. The peak concentrations of E<sub>2</sub> achieved and sustained for 2 hours with subcutaneous

administration of 100 µg/ml with this formulation were 2000 pg/ml (7 nM). We assume that the 1 mg/ml dose of E<sub>2</sub> would produce 10 times higher peak concentrations (or 70 nM). In a variety of *in vitro* studies these peak concentrations of E<sub>2</sub> are potently neuroprotective and do not exhibit toxicity [2,3,10].

Our observation of a therapeutic window for E<sub>2</sub> administration in an oil formulation of 6 hours for a third of animals tested indicates that estrogens can protect brain tissue through mechanisms that are initiated long after the onset of the ischemic event. A variety of mechanisms for estrogen neuroprotection have been proposed, among which are activation of anti-apoptotic proteins [1,7,14,28] and preservation of mitochondrial integrity [4,27]. Our results suggest that estrogen treatment delayed by as much as 6 hours can affect these neuroprotective processes in a way that affords substantial benefit for a subpopulation of subjects. Although it is uncertain whether the therapeutic window of estrogen could be extended beyond 6 hours, this 6-hour time frame is clinically relevant in that it is logistically difficult to institute therapy in many patients with acute stroke at earlier times.

It is uncertain why some subjects responded, while others didn't, to the E<sub>2</sub> treatment when it was administered at 6 hours after the onset of cerebral ischemia. This model and treatment paradigm is significant for its potential clinical relevance. Our study indicated that this model could be used to determine the mechanism contribute to the different response of each subject to the therapeutic intervention, which frequently happens in clinical circumstance.

We observed cortical as well as subcortical protection with delayed E<sub>2</sub> treatment. In pretreatment paradigms, consistent protection of the cortex has been reported [22,23]. On the other hand, subcortical protection of E<sub>2</sub> has been reported by some studies [20,29], while others do not [6,8]. The apparent resistance of the subcortex to E<sub>2</sub> protection could be due to the lack of estrogen receptors (ER) expression at the basal ganglion [24]. This could contribute to the relative resistance of the basal ganglion to E<sub>2</sub> treatment through the lack of ER-mediated neuroprotection in this region of the brain, as has been proposed. However, the basal ganglion is an exclusive territory of the middle cerebral artery and as such does not receive blood flow from collateral arteries, in contrast to the extensive collateral circulation of the cortex [22]. As such, MCAO results in a more severe ischemia in the subcortex than the cortex [8,19]. Our observation that high doses of E<sub>2</sub>, even when administered after the onset of the ischemia can protect the subcortex supports the hypothesis that this brain region is estrogen protectable.

In conclusion, we have shown that a subcutaneous oil preparation for E<sub>2</sub> delivery that produces rapid and sustained elevations in circulating E<sub>2</sub> causes a dose-dependent protection against focal cerebral ischemia. This protection is seen for as long as 6 hours after the onset of ischemia. Collectively, these results suggest that subcutaneous estrogen treatment with oil formulations during ongoing strokes may improve neurological outcome.

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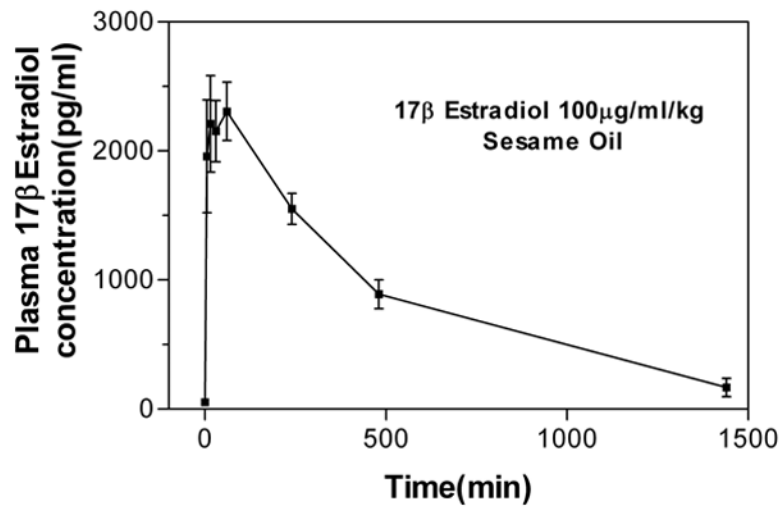
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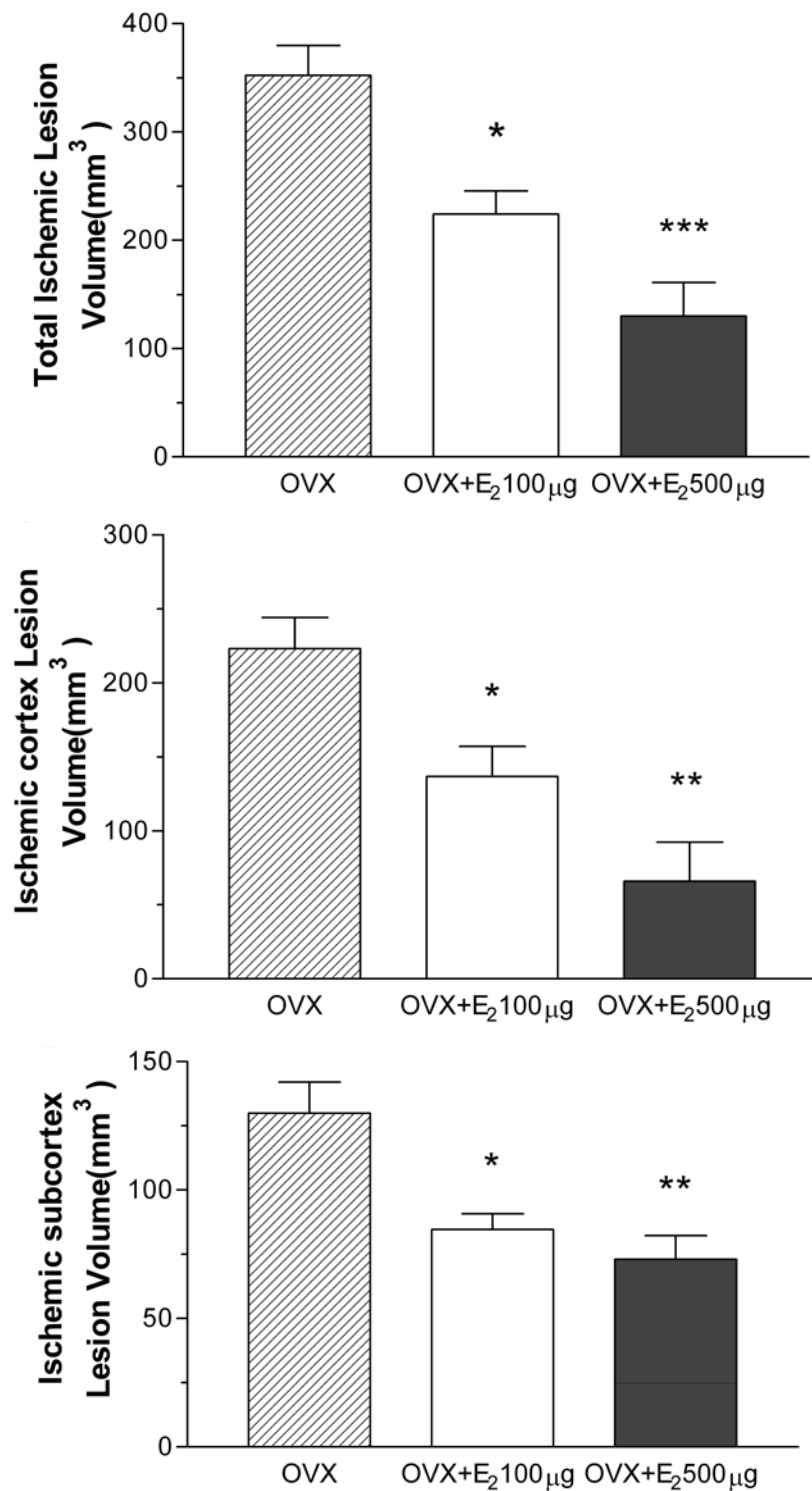
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**Figure 1.**

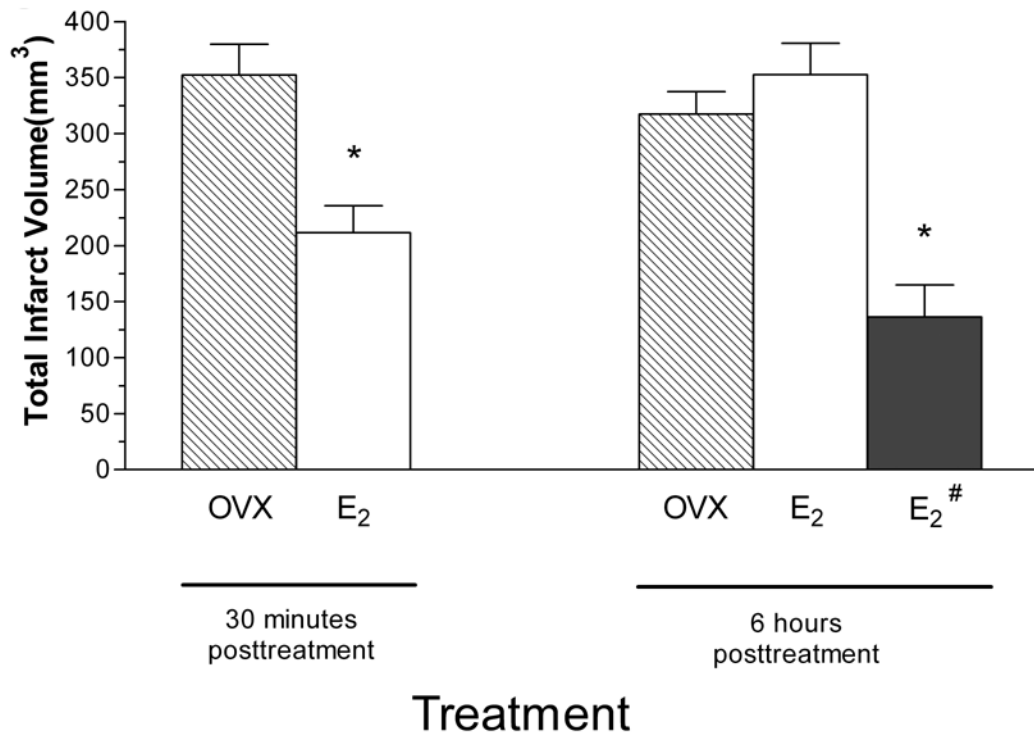
Plasma estradiol levels after  $E_2$  administration.  $E_2$  was subcutaneous administered at the dose of 100  $\mu\text{g}/\text{kg}$  in 7 ovariectomized rats. Blood samples were taken prior (time 0), and 5, 15, 30 minutes, and 1, 4, 8, 24 hours after  $E_2$  administration. Plasma estradiol concentration is given in pg/ml: 1nM = 272 pg/ml.





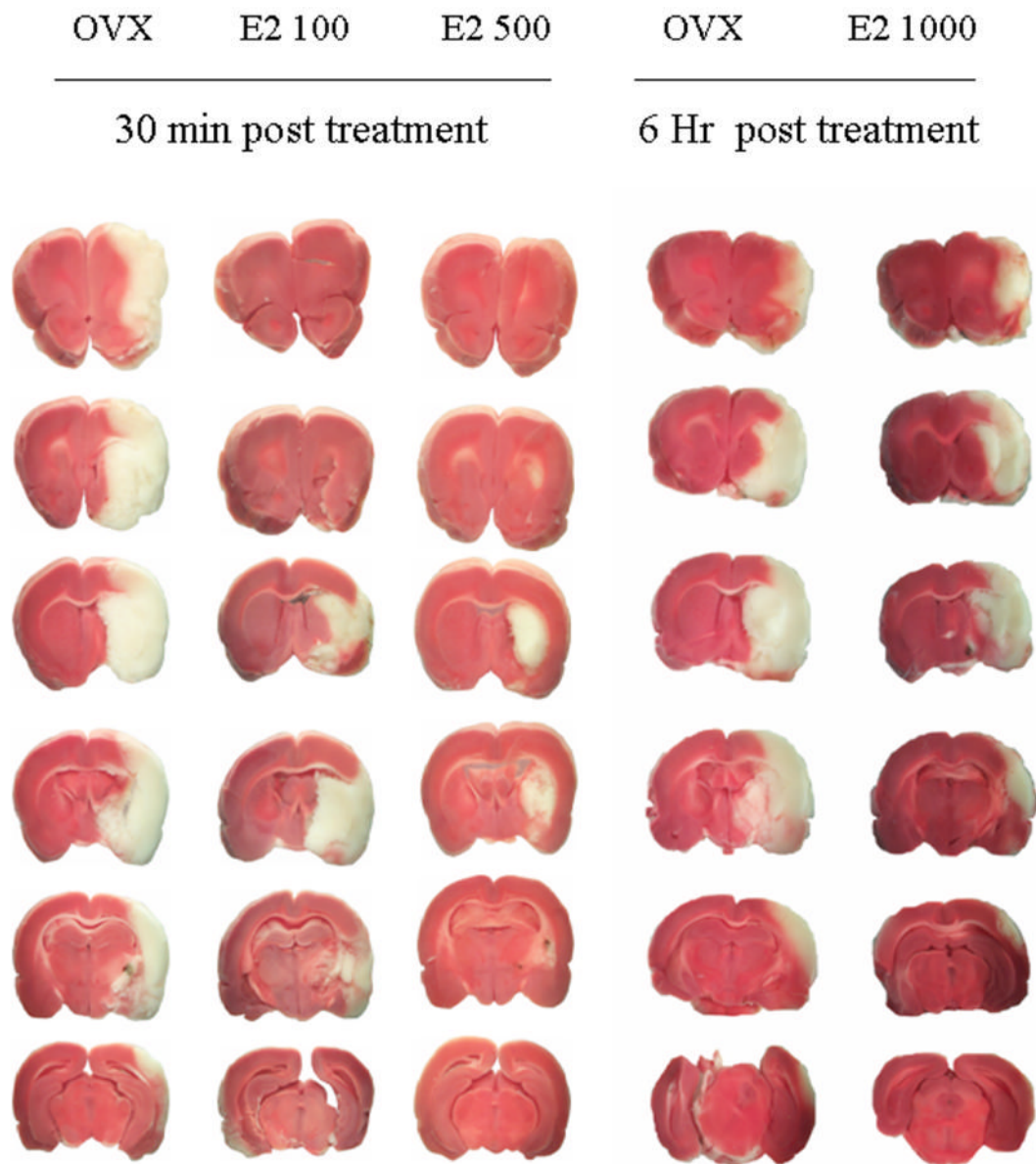
**Figure 2.** Effect of 30 minutes posttreatment with 100 or 500 µg/kg of E<sub>2</sub> on total lesion volume (A), cortical lesion volume (B), and subcortical lesion volume (C) induced by p-MCAO. E<sub>2</sub> significantly decreases total lesion volume by 36.4% ± 6.0% and 63.1% ± 8.9% in 100 µg/kg (n=5) or 500 µg/kg (n=7) group vs. OVX group (n=9), respectively. E<sub>2</sub> significantly decreases

cortical lesion volume by  $38.8\% \pm 9.2\%$  or  $70.5\% \pm 11.8\%$  compared to OVX rats in 100  $\mu\text{g}/\text{kg}$  (n=5) or 500  $\mu\text{g}/\text{kg}$  (n=7) group vs. control group (n=9), respectively.  $\text{E}_2$  significantly decreases subcortical lesion volume by  $35.3\% \pm 4.7\%$  or  $43.8\% \pm 7.2\%$  compared to OVX rats in 100  $\mu\text{g}/\text{kg}$  (n=5) or 500  $\mu\text{g}/\text{kg}$  (n=7) group vs. control group (n=9), respectively. \* $P < 0.05$  vs. control. \*\* $P < 0.01$  vs. control. \*\*\* $P < 0.001$  vs. control



**Figure 3.**

Effect of 30 minutes or 6 hours posttreatment with 1 mg/kg of E<sub>2</sub> on ischemic total lesion volume induced by p-MCAO. When administered at 30 minutes after occlusion, E<sub>2</sub> (n=6) significant decreases the total infarct volume by  $40.0\% \pm 6.9\%$  vs. OVX group (n=7). When administered at 6 hours after occlusion, protective effect of estradiol was demonstrated in 4 of 12 rats (E<sub>2</sub><sup>#</sup> group), with a  $57.0 \pm 9.0\%$  reduction of the total infarct volume vs. OVX group (n=8), while, no protective effect was identified in the other 8 rats (E<sub>2</sub> group). \*P<0.05 vs. control.



**Figure 4.** Photograph displaying representative brain sections obtained from ovariectomized rats treated with E<sub>2</sub> at 30 minutes or 6 hours after MCA occlusion. Rat were treated with vehicle (OVX), or E<sub>2</sub> at the dose of 100 µg/kg (E2 100), 500 µg/kg (E2 500), or 1000 µg/kg (E2 1000).